DETERMINATION OF FORMALDEHYDE AND GLUTARALDEHYDE BOUND TO COLLAGEN BY CARBON-14 ASSAY*

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ABSTRACT

The difficulties associated with the quantitative determination of aldehydes bound by collagen and the assessment of the reliability of the methods used are discussed. Model tanning experiments have been carried out using ^{14}C-labelled glutaraldehyde and formaldehyde, and the aldehyde bound determined by ^{14}C assay. With glutaraldehyde it has been found that the amounts present in the tanned material after drying are about 70–80 percent of those estimated from losses of aldehyde from the solution. The amounts bound by the dry material vary from 1.3 percent following tannage at pH 4.0 to 6.0 percent at pH 8.0. At the high pH values the amounts bound exceed a 1:1 ratio with the amino groups indicating that polymerization of the aldehyde has occurred. Recovery of lysine from acid hydrolysates is reduced following glutaraldehyde tannage and it is suggested that such losses may serve as indication of the degree of reaction with ε-amino groups.

It has been shown that it is possible to recover all the formaldehyde from freshly tanned collagen by steam distillation from acid. Full recovery is also possible after heating dry at 100°C. Heating in boiling water or treatment in acid lead to large losses of formaldehyde, but a small amount becomes firmly fixed and is no longer recoverable by chemical means.

INTRODUCTION

The quantitative determination of aldehydes bound to collagen in tanning has always presented difficulties, firstly because of the relatively small amounts involved, less than one percent in the case of formaldehyde, but more particularly because of the uncertainty regarding the complete recovery of the aldehyde from

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the protein, see for example Nitschmann, Hadorn and Lauener (1). Any chemical determination must depend on the quantitative breakdown of the bond between the aldehyde and the protein without destruction of the characteristic properties of the aldehyde, preferably followed by its separation from the protein. With formaldehyde it has generally been assumed that steam distillation from 2*N* sulfuric acid or straight distillation from phosphoric acid gives complete recovery. This appears to be true when formaldehyde is added to collagen in a closed vessel and kept under tanning conditions for 24 hours before distilling the whole contents of the flask (2). Whether recovery is still complete after the leather has been dried out and aged cannot be certain and with volatile aldehydes it is difficult to test this.

With less volatile aldehydes quantitative recovery by distillation of the aldehyde alone is often incomplete and no entirely satisfactory method has yet been suggested for their direct determination in leather. In an extensive investigation of the tanning action of glutaraldehyde, workers at the Eastern Regional Laboratories of the U. S. Department of Agriculture (3–9) have relied on estimating uptake from the loss of aldehyde from the solution. This approach is subject to various disadvantages, namely, (1) errors due to dilution by the water in the pelt, (2) the necessity to assume that the aldehyde is evenly distributed between the external solution and the solution in the pelt and that there is no change in this distribution during subsequent washing, or loss of aldehyde during drying of the tanned skin, and finally, (3) the difficulty of making adequate allowance for any polymerization of the aldehyde during the tanning. The above workers took steps to eliminate as far as possible the errors associated with the first and last of these. Direct determination of the aldehyde fixed by difference following analysis for hide substance, moisture, fat, etc., is tedious and is, at best, only approximate since the amounts involved are generally rather small.

In view of these considerations it was decided to determine the fixation of glutaraldehyde under various conditions by a radio-isotope technique and see how the values so obtained agreed with those estimated by difference methods; also, to assess the stability of the aldehyde-protein bond by determining the amounts of aldehyde remaining after various treatments.

At the same time the recovery of formaldehyde by steam distillation was reinvestigated using ^{14}C-labelled formaldehyde. This work was undertaken because recent work on the stability of formaldehyde leather indicated that following heating or storage under warm moist conditions recovery was no longer complete (10). In particular it has been found that although the formaldehyde content as determined chemically is reduced considerably by storage there is no corresponding increase in the number of amino groups available to fluorodinitrobenzene suggesting that some aldehyde has become irreversibly bound. The fact that the leather was apparently unaffected, whereas untanned skin stored under the same conditions was badly damaged, reinforced this hypothesis.

EXPERIMENTAL

Raw Material.

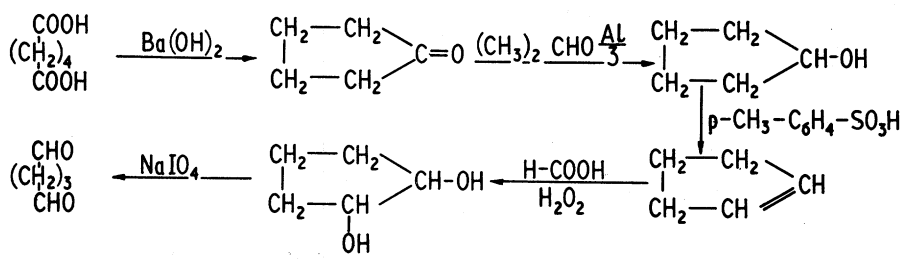
Kangaroo tail tendons were dissected out of the tails as soon as possible after death, washed in several changes of 5 percent sodium chloride and dehydrated with acetone.

Skin pieces were prepared from commercial degreased and pickled Cape type sheepskins. The skins were depickled in sodium acetate, washed and dehydrated with acetone.

^{14}C-labelled adipic acid for synthesis of glutaraldehyde and ^{14}C-labelled formaldehyde were obtained from the Radiochemical Centre, Amersham.

Synthesis of Glutaraldehyde.

The method chosen was selected on the grounds of availability of a suitably labelled starting material and the relative simplicity of the manipulative procedures involved (11-16).



From preliminary work on the various stages of the synthesis using inactive material it was estimated that the overall conversion of adipic acid to glutaraldehyde would be between 40 and 50 percent. Unfortunately when repeated on a smaller scale with ^{14}C-labelled adipic acid the yield was much less. The ^{14}C-glutaraldehyde was steam distilled from the final mixture and a 0.4 percent solution obtained.

Preparation of Tanned Samples.

Glutaraldehyde.—Acetone dehydrated tendons or skin in the form of pieces of sheepskin about 0.5 cm. square were soaked back in water, drained and tanned in the appropriate solutions, using 10 ml. per gram dry weight for 24 hours at 20°C. All tanning solutions contained 10 percent Na_2SO_4 and pH values were adjusted to the required values with acetate, pH 4.0, phosphate, pH 5.0 and 6.5 and bicarbonate, pH 8.0.

The concentrations of glutaraldehyde[1] used corresponded to 3 and 12 percent on dry weight of tendon. After tannage the samples were washed in four changes of water over a period of 24 hours and dehydrated with acetone.

[1]All concentrations refer to pure glutaraldehyde (100%).

Formaldehyde.—With formaldehyde only one set of conditions was used, : percent on dry weight of tendon or skin at pH 8.0. Two parallel tannages were carried out, one with active formaldehyde and another on a larger scale with inactive material to provide a relatively large amount of tanned skin for chemical determinations. The tanned material was washed and dried as above.

Methods.

Determination of Carbon-14—Solutions were blended directly with NE213 liquid scintillator (Nuclear Enterprise) using hyamine salt.

Samples, 50–120 mg., of tendon or skin were burnt in oxygen in a sealed three-necked 500 ml. flask. Pressure was slightly reduced and the sample ignited by a high voltage spark discharge. A known weight of the scintillation counting liquid, PPO/POPOP,[2] containing 2-phenylethylamine to absorb CO_2 was injected into the flask (17). After half an hour 5 ml. were transferred to a glass vial, weighed and left in the glove box for 18 hours before counting. Tests indicated that combustion was complete.

Counting was carried out using a modified Isotope Developments Ltd. photomultiplier tube Type 653A contained in a lightproof glove box. A glass well with a ground glass base was mounted on the tube in place of the sodium iodide crystal, optical contact between the base and the tube being made by a film of silicone fluid. The glass vials containing the solution to be counted were placed in silicone fluid contained in the glass well. An internal standard of ^{14}C-hexadecane (CFR 5, Radiochemical Centre) was used to check counting efficiency.

Glutaraldehyde was determined chemically in solutions by volumetric estimation of iodine consumed in the alkaline oxidation of the bisulfite complex (3, 18)

Formaldehyde.—For the chemical determination of formaldehyde in skin, samples usually 1 to 2 g., were steam distilled from 2*N* H_2SO_4, 500 ml. distillate collected and the formaldehyde determined as the dimedone derivative (2, 19).

Amino Acid Analysis.—Samples were hydrolyzed in 6*N* HCl for 20 hours at 102–105°C., the acid removed by evaporation on a steam bath and the residues made up to a suitable volume. Separations were carried out by the procedures described by Hannig (20) using a Bender and Hobein automatic analyzer.

Cross-Links were determined by the stress-strain technique as described by Cater (21).

RESULTS

Reaction of Glutaraldehyde with Collagen.

As the yield of ^{14}C-labelled glutaraldehyde was low, the amounts available for experiments were limited. Whenever possible, therefore, both counts and chemical

[2] 2,5 diphenyloxazole/1,4-di [2,5(phenyloxazolyl)]-benzene.

determinations were made on original and used liquors in order to obtain as accurate an estimate as possible of the relationship between counts and glutaraldehyde concentration as determined by iodometric titration. The ratio was found to be slightly higher with the used than with the unused liquors; this could be due to low values for the chemical determination associated with polymerization of the aldehyde during tanning. In view of the results of Fein *et al.* (3) and the fact that there was the same proportional increase at pH 4.0 as at pH 8.0 it was considered that it was more likely due to the presence of about 10 percent of a radiochemical impurity not taken up by the tendon. Allowance was made for this in deriving the factor for conversion of counts to glutaraldehyde.

The amounts of glutaraldehyde bound as estimated from losses from the solution and by direct ^{14}C counting of the incinerated tendons are given in Table I, together with values for the cross-links introduced. The amounts of glutaraldehyde taken up increase with pH and concentration as in earlier investigations (3, 21). In most cases the amounts actually bound are between 70 percent and 80 percent of those estimated by loss from solution. Some adsorption or loose association of the aldehyde with the skin must therefore occur, this loosely bound material being removed in the subsequent washing and drying of the tanned collagen. With the lower concentration of aldehyde, exhaustion of the liquors at the higher pH values is virtually complete, and up to 80 percent of the aldehyde offered is firmly bound. Quadrupling the amount offered approximately doubles fixation, the bound aldehyde approaching a maximum of about 5.0 percent.

TABLE I

UPTAKE OF GLUTARALDEHYDE BY COLLAGEN (KTT) AS DETERMINED BY CARBON-14 ASSAY

Conditions of Tannage		Aldehyde Bound[1,2]				
		By loss from solution				
pH	Aldehyde on air dry wt. %	Chemical g.	^{14}C Assay g.	By Direct ^{14}C Assay on tanned tendon	Direct estimate from loss of solution %	Cross-links Moles per 10^5 g.
4.0	3.0	1.8	1.7	1.3	76	3
5.0	3.0	2.2	2.3	1.7	74	4
6.5	3.0	3.0	2.7	2.3	85	6
8.0	3.0	3.2	3.1	2.4	77	8
4.0	12.0	3.4	3.5	2.8	80	8
5.0	12.0	4.3	4.7	3.2	68	6
6.5	12.0		5.4	4.3	80	11
8.0	12.0		6.5	4.8	74	13

[1]Per 100 g. moisture-free weight.

[2]The molecular weight of glutaraldehyde is 100, therefore these values multiplied by 10 gives moles per 10^5 g.

The results of stability tests presented in Tables II and III show that little glutaraldehyde is lost on boiling in water for 7 hours and the decrease in cross-links is also small. The result for the tendon tanned with the lower concentration at pH 8.0 is anomalous, the decrease in cross-links being much greater than is general, both in this and other series of experiments. Treatment in normal sulfuric acid causes some reduction in glutaraldehyde content in the first few hours but no further decrease on longer exposure. The original glutaraldehyde content of the tendon used in this test was higher than normal, and it seems probable that the initial decrease is due to the removal of loosely bound aldehyde which had not been completely washed out.

TABLE II

STABILITY OF GLUTARALDEHYDE-COLLAGEN BONDS TO BOILING WATER

Conditions of tannage		Moles per 10^5 grams moisture free tendon			
		Aldehyde Bound ^{14}C Assay		Cross-links per 10^5 g.	
pH	Aldehyde offered dry wt. %	Initial	After 7 hrs. in boiling water	Initial	After 7 hrs. in boiling water
4.0	3.0	13	13	3	2
5.0	3.0	17	15	4	3
6.5	3.0	23	19	6	4
8.0	3.0	24	25	8	4
4.0	12.0	28	26	8	6
5.0	12.0	32	32	6	7
6.5	12.0	43	38	11	10
8.0	12.0	48	43	13	11

TABLE III

STABILITY OF GLUTARALDEHYDE-COLLAGEN BONDS TO ACID

	Moles per 10^5 g. moisture free tendon	
	Aldehyde Bound	Cross-Links
Tendon tanned at pH 8.0 12% Aldehyde Offered	64	8
After treatment in N H_2SO_4		
for 2 hr.	54	8
24 hr.	56	7
120 hr.	54	9

In a further experiment the tendons used for the determination of cross-links were analyzed for amino acid composition. In contrast with formaldehyde tanned tendon there was no loss of tyrosine (10) and the only changes observed were losses of lysine and hydroxylysine (see Table IV). These losses ran parallel with the glutaraldehyde content of the tendons suggesting that they may serve as a measure of the extent of the reaction between glutaraldehyde and collagen. So far neither the glutaraldehyde-lysine derivatives nor their breakdown products have been identified on the Moore and Stein chromatographic columns. Attempts are being made to do this by other means.

TABLE IV

REACTION OF GLUTARALDEHYDE WITH KTT COLLAGEN
Moles per 10^5 g.

pH of tannage	Aldehyde Bound ^{14}C Assay	Cross-links	Amino Groups		Distribution of Reacted	
			Free	Reacted	In Cross-links	In Unipoint Fixation
Untanned	0	0.2	30.8	—	—	—
Tanned at 4.0	21	4.9	14.9	15.9	9.4	6.5
5.0	24	7.5	11.1	19.7	14.6	5.1
6.5	32	8.0	8.2	22.6	15.6	7.0
8.0	38	8.3	8.0	22.8	16.2	6.6

Reaction of Formaldehyde with Collagen.

In order to economize on active material two parallel tannages were carried out, one with ^{14}C-labelled formaldehyde and another on a larger scale with inactive material for chemical determinations. Chemical determinations made on the air-dry tanned collagen indicated that the uptake of formaldehyde was the same in each of these.

The results obtained from ^{14}C counting are considered first. The amounts fixed vary between 0.7 and 1.0 percent corresponding to 23 to 33 moles per 10^5 g., i.e., approximating one mole of formaldehyde per amino group. In three out of the four experiments heating at 100°C. for 24 hours caused a small decrease of 10–15 percent. Both boiling in water and treatment in normal acid caused more substantial decreases in the aldehyde content. These losses were accompanied by the expected decrease in the number of cross-links (Table V, Expt. IV). The possibility that heating dry might lead to an increase in the proportion of formaldehyde firmly bound and hence to reduced losses on boiling or treatment with acid was examined. No such effect could, however, be demonstrated.

TABLE V

COMPARISON OF FORMALDEHYDE DETERMINATIONS BY CHEMICAL AND ISOTOPIC TRACER METHODS

	Formaldehyde Bound			
	g. per 100 g. air dry leather		Millimoles per 100 g.	
	Chemical	^{14}C Assay	Chemical	^{14}C Assay
Experiment I				
As tanned	1.05	0.98	35.0	32.7
Heated at 100°C., 24 hr.	0.78	1.08	26.0	39.3
Boiling water, 5 hr.	0.09	0.31	3.0	10.3
N H_2SO_4, 48 hr.	0.33	0.55	11.0	18.3
Experiment II				
As tanned	—	0.74	—	24.7
Heated at 100°C., 24 hr.	—	0.69	—	23.0
Heated at 100°C., 24 hr., followed by boiling for 5 hr.	0.06	0.40	3.0	13.3
Stored over water at 40°C., 2 weeks	0.43	0.70	14.3	23.3
Experiment III				
As tanned	0.81	0.81	27.0	27.0
Heated at 100°C., 24 hr.	0.71	0.74	23.7	24.7
Boiling water, 5 hr.	0.04	0.22	1.3	7.3
Heated at 100°C., 24 hr., Boiling water, 5 hr.	0	0.26	0	8.7
N H_2SO_4, 48 hr.	0.48	0.61	16.0	20.3
Heated at 100°C., 24 hr., *N* H_2SO_4, 48 hr.	0.51	0.62	17.0	20.7
Stored over water at 40°C., 6 weeks	0.34	0.31	—	—
Experiment IV — Tendons			Cross-links, Moles per 10^5 g.	Moles per 10^5 g.
As tanned	—	0.68	5.6	22.7
Heated at 100°C., 24 hr.	—	0.53	5.9	17.7
Boiling water, 2 hr.	—	0.20	1.0	6.7
Boiling water, 5 hr.	—	0.12	0.5	4.0
N H_2SO_4, 24 hr.	—	0.34	3.3	11.3
N H_2SO_4, 48 hr.	—	0.30	2.6	10.0

With the air-dry material, recovery of formaldehyde by steam distillation was found to be complete, the results agreeing well with those obtained from ^{14}C counting. Heating at 100°C. had no effect on recovery but after boiling in water or treatment in acid the amounts found by chemical means were appreciably lower than by the isotope method indicating that a proportion of the formaldehyde had become firmly bound to the protein. About 0.2 percent formaldehyde on protein weight or 7 moles per 10^5 g. appears to be involved in this way.

In the first test, storage over water at 40°C. for 2 weeks led to no actual loss of formaldehyde though the amount recoverable by steam distillation was reduced by over 40 percent. In a second test in which storage was extended to 6 weeks both methods indicated losses of the same order.

DISCUSSION

As expected the amount of glutaraldehyde actually bound by the collagen was found to be lower than that estimated from analyses of the tanning solutions, about 20–30 percent apparently being only loosely held and washed out of the leather after tanning. In cases where the exhaustion approaches completion, analysis of the liquor probably gives a reasonable estimate of the amounts bound provided, as in the present work, a reasonably accurate estimate of the water carried into the system by the pelt can be made.

The glutaraldehyde bound approaches a maximum of about 5 percent or 50 moles per 10^5 g., thus exceeding a one to one ratio with the amino groups. The amount of aldehyde bound in excess of one mole per cross-link and one mole per remaining amino group increases steadily with the pH of tannage. It seems unlikely that the guanidino groups of the protein will react appreciably with glutaraldehyde even at the highest pH value, and condensation of the aldehyde with itself appears to be the more likely explanation of the excess, particularly in view of the recent work reported by Aso and Aito (22), who cite evidence for the formation of oligomers containing 3 to 4 aldehyde units.

The stability tests indicate that little glutaraldehyde is lost by treatment in boiling water or in normal acid, therefore, such condensation products if formed must be insoluble, or what is more likely are actually bound by the protein. On this basis the amounts of glutaraldehyde bound indicate that, on the average, products containing two or three moles of glutaraldehyde are formed. It also follows that the bond linking the monomers is at least as stable as that involving the amino group of the protein. It is probable that the superiority of glutaraldehyde over other aldehydes as a cross-linking agent is associated with this ability to condense to form small oligomers. Further work on glutaraldehyde and glyoxal is being undertaken to elucidate this point.

Loss of lysine and hydroxylysine appears to offer a possibility of assessing the extent of the reaction of glutaraldehyde with collagen. On this assumption it seems that only about 75 percent of these ε-amino groups are available for reaction leaving about 8 moles per 10^5 g. free. In other experiments also the recovery of free lysine and hydroxylysine has approximated similar limiting values and preliminary attempts at the formol titration indicate a similar proportion of unreacted groups. At all pH values the number of amino groups lost exceeds the number involved in cross-links by some 5 to 7 moles per 10^5 g. indicating that appreciable amounts of glutaraldehyde are unipointly fixed. Before any definite conclusions can be drawn, however, it must be established that there is no breakdown of amino-aldehyde bonds during hydrolysis, or that, if this does occur, there is no liberation of the free amino acid.

With formaldehyde the use of Carbon-14 labelled aldehyde has established that steam distillation from acid leads to satisfactory recovery from freshly tanned material. In contrast to glutaraldehyde, the amounts bound correspond to less than one mole per amino group, and there is no need, therefore, to postulate condensation.

With regard to stability, heating dry causes some loss of formaldehyde, but there is no indication that recovery is reduced due to formation of more stable links.

Stability to boiling water and acid is relatively poor, most of the formaldehyde being lost. A small proportion of the formaldehyde, corresponding to about 5–10 moles per 10^5 g. becomes more firmly fixed following boiling in water or treatment in acid and is no longer recoverable by steam distillation. Cross-links are, however, reduced to about one so that most of this must be unipointly fixed, possibly by tyrosine residues since this amino acid is completely lost following formaldehyde tanning. The instability of the cross-links is apparently not only associated with the aldehyde-amide or guanidine bonds as previously suggested (21) but also involves breaking of the bond with the amino group. The lower stability of this bond as compared with that formed with glutaraldehyde is also illustrated by the almost complete recovery of lysine and hydroxylysine from formaldehyde tanned collagen.

Unfortunately the results throw little light on the effects of moist storage on the binding of formaldehyde (10).

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